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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

(Small Entity)

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (b)(2).

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INVENTOR(s)/APPLICANT(s)

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TITLE OF THE INVENTION (280 characters max)

Quantitative Assay Method For Detecting Abasic Sites And Anylyzing DNA Repair In Genomic DNA

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ENCLOSED APPLICATION PARTS *(check all that apply)*

- [X] Specification — *Number of Pages* 28
- [X] Drawing(s) — *Number of Sheets* 15
- [X] Small Entity Statement
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METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT *(check one)*

- [X] A check or money order is enclosed to cover the filing fees
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States

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- [ ] Yes, the name of the U.S. Government agency and the Government contract number _____

Respectfully submitted,

SIGNATURE _____ Date December 21 1999

TYPED or PRINTED NAME Lawrence Harbin REGISTRATION NO. *(if appropriate)* 27,644

- [ ] Additional inventors are being named on separately numbered sheets attached hereto

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PROVISIONAL PATENT APPLICATION

Title: QUANTITATIVE ASSAY METHOD FOR DETECTING ABASIC SITES AND ANALYZING DNA REPAIR IN GENOMIC DNA

Filed: December 21, 1999

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The Following Papers Are Attached:

(1) Rapid Simple and Sensitive Quantitative Detection Assay for Abasic Sites in DNA,
(2) A Simple and Rapid Non-Isotopic Microtiter Plate-Based Chromogenic Assay for Quantitative Detection of Abasic (AP) Sites in DNA,
(3) Quantitative Detection of Abasic (AP) Sites in DNA and Analysis of DNA Base Modification and Repair by an Improved Aldehyde Reactive Probe (ARP) Assay, and
(4) Rapid Assay for DNA Damage and Repair Using Silicon Microstructures

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APPLICATION FOR PATENT OF AN INVENTION

Rapid Simple and Sensitive Quantitative Detection Assay f or Abasic Sites in DNA.

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ABSTRACT

A simple, rapid, sensitive, and cost-effective assay method requiring no radioactivity is provided for detecting and qauntitating abasic site in DNA wherein the method involves DNA binding to microtiter plate, a chemical reagent probe labeling, and a colorimetric analysis. Utilized in the method are isolated DNA from cells or tissues, DNA binding agent to the plate and avidin-biotin conjugated mechanism of colorimetric analysis.

Significance

The current development is worthy of patent, being unique and greatly significant to provide rapidity, sensitivity, simplicity and cost-effectiveness than any other ever presented in accurately detecting and quantifying abasic sites in human genomic DNA as indication of genomic instability and throughput analysis of oxidative stress in large samples for clinical application and populational studies. Several methods for quantifying abasic (AP) sites in DNA have been reported and only a few allow discrimination of low AP sites in DNA, but these methods require radioactivity, which endangers the health of the operators and contaminates the environment. Others not requiring radioactivity are either extremely time consuming, technique-sensitive, cumbersome and not cost-efficient. A previous method attempting a solution to these challenges used Aldehyde Reactive Probe (ARP) to tag biotin to the aldehyde group of AP sites in DNA (Kubo et al, 1992). The ELISA-like method was limited in its sensitivity, in addition to the required laborious step of ethanol precipitation/centrifugation to remove excess ARP that limits the use of the assay when available DNA sample is small. The current development allows rapid and sensitive measurement of AP sites in DNA directly on the microtiter plate, obviating the need for ethanol precipitation or ultrafiltration/centrifugation. Above all, there is no assay at present that can be automated for quantitative detection of abasic sites in large samples of DNA as indicated in the current development. The current innovation therefore becomes significant and unique in combining these features:

1. Simplicity
2. Rapidity
3. Possible automation for large samples
4. Non-radioactive method and Environmental friendly
5. Able to quantitatively detect specific base damage in a spectrum of base modifications
6. Most cost-effective
7. Clinical application and population studies

Description

Filed of the Invention

The present invention relates to novel methods for rapid, sensitive and cost-effective detection of DNA damage. More particularly, the present invention relates to enhanced DNA immobilization to microtiter plate and reaction with abasic site-specific aldehyde-group reactive biotinylated chemical agent in providing methods for identifying and quantifying abasic sites in DNA to estimate level of oxidative stress/injury.

Background of the Invention

Free radicals, or reactive oxygen species, are generated in vivo by a variety of mechanisms that include oxidative stress, redox cycling, and ionizing radiation. Several cellular processes can endogenously generate superoxide anions, which could in turn generate hydroxyl radicals in the nucleus of the cell, which then cause formation of abasic (AP) sites resulting from the removal of purine or pyrimidine bases and site specific damage to DNA. Depending on the mechanism for generating the free radicals, free radical induced oxidative DNA base modifications include 8-hydroxy-guanine, thymine glycol, formamido residues, dihydrothymine, 5-hydroxymethyl uracil, and 5-hydroxy-5-methylhydantoin. The base excision repair pathway (BER) repairs these base modifications. In its initial step, the BER pathway results in the formation of abasic (AP) sites. Deficiency in the DNA base excision repair pathway (BER) and increasing oxidative stress could contribute to increased background levels of AP sites in cells and tissues.

Interestingly, recent studies implicate increased background level of oxidative DNA damage in the pathogenesis of some human diseases. These include Alzheimer's disease (Mecocci et al., 1994, Ann Neurolog. 36:747-651; Prashad et al., 1996, Proc. Natl. Acad. Sci. USA 93:5146-50), amyotrophic lateral sclerosis (10), Parkinson's disease (11), cataract formation, aging process (12), radiation exposure (Wilson et al., 1992, Cancer Res. 48:2156-2162) ischemic damage and stroke (1996, NIH Guide 25), metal toxicity 9carmichael et al., 1995, Mutat. Research 326:235-43), breast cancer (Djuric et la., 1996, Cancer 77:691-6), carcinogenesis (Ames et al., 1995, Proc. Natl. Acad. Sci. USA 92:5258-65). Molecular mechanisms, including oxidative DNA damage and alteration of a cell's ability to repair damaged DNA, may lead to the development of genomic instability. Genomic instability is believed to occur in an early step in the process of carcinogenesis. In addition, cells and human tissues are being screened for specific DNA damage in order to correlate the action of DNA damaging agents with human diseases and verify the contribution of DNA damaging agents in specific genetic states to manifestation of human diseases

There is a need for sensitive assays for specific lesions resulting from oxidative DNA damage. It is known to those skilled in the art that exposure of an individual to ionizing radiation or certain chemotherapeutic agents or toxic agents can result in oxidative DNA damage. Thus, there is a need for means, such as the methods and compositions according to the present invention, which are clinically useful to assess exposure to toxic agents, chemotherapy or radiation therapy. In addition, for individuals at high risk of a pathological condition associated with oxidative DNA damage, it may be useful to monitor the levels of abasic site and specific DNA base modifications resulting from oxidative DNA damage prior to clinical onset of the condition. Although several methods for quantifying AP sites have been reported, some of these allow discrimination of low AP sites in DNA, but require radioactivity. Others while not requiring radioactivity are time consuming,

technique-sensitive and cumbersome. In addition, with the exception of thymine glycol which can be measured using an immunoassay (Hubbard-Smith et al., 1992, Radiat. Res. 130:160-5; U.S. Pat. No. 5,552,285) or by mass spectrometric analyses (Markey et al., 1993, Ann. NY Acad. Sci. 679:352-7), quantitation of each specific DNA base modification has been hindered by the lack of sensitivity of existing assays to detect biologically significant levels of each modification amongst the variety of base modifications that may be present.

Summary of the Invention

The present invention is directed to methods, compositions and development of kits to facilitate accurate, rapid and cost-effective measurement of abasic (AP) sites and DNA base modifications in the genomic DNA of cells and tissues. Measurements of abasic (AP) sites in genomic DNA are carried out directly and completely on the microtiter plate. In one embodiment, the method of the present invention comprises innovatively derived ratio of isolated genomic DNA from cells and tissues and a DNA coating agent resulting in a unique enhanced binding of genomic DNA to the microtiter plate. The bound aldehyde group in the abasic site in the DNA was specifically labeled/tagged by an excess of a biotinylated aldehyde reactive chemical reagent. In order to prevent contributory non-specific sites from excess reagent, on the microtiter plate, the excess reagent was innovatively removed without detaching the bound DNA. The abasic (AP) sites contained in the tagged DNA were then quantitatively detected chromatographically by an avidin-biotin conjugate method in the microtiter plate.

Measurements of a specific DNA base modification are carried out at the dimer level. The method of the present invention comprises a complete N-glycosylase (endonuclease) enzyme digestion resulting in the production of abasic site in DNA. In the ensuing step, using similar method as above, a unique enhanced binding of genomic DNA to the microtiter plate was achieved. The bound aldehyde group in the abasic site in the DNA was specifically labeled/tagged by an excess of a biotinylated aldehyde reactive chemical reagent. In order to prevent contributory non-specific sites from excess reagent, on the microtiter plate, the excess reagent was innovatively removed without detaching the bound DNA. The abasic (AP) sites contained in the tagged DNA were then quantitatively detected chromatographically by an avidin-biotin conjugate method in the microtiter plate. Since certain DNA base modifications prevent or significantly inhibit the hydrolysis of the neighboring phosphodiester bond by an endonuclease which hydrolyzes single stranded DNA, the sample of DNA to be assayed for the presence of DNA base modifications is digested with the endonuclease; and then with a phosphatase which dephosphorylates terminal phosphate groups. An internal DNA standard, similar to the samples, with specific inclusion of base modification was similarly digested and the resulting abasic sites determined as described for accurate and sensitive quantitation. In an additional aspect of the present invention, provided is an assay kit for a method to detect and quantify abasic (AP) site in genomic DNA sample isolated from cells and tissues. When this method was N-glycosylase (endonuclease) enzyme-coupled, detection and quantitation of DNA base modifications selected from the group consisting of 5-hydrox-5-methylhydantoin, 5-hydroxymethyluracil, or 8-hydroxyguanine was made possible. The detailed description of the present invention has been done for purposes of illustration. In view of the descriptions and illustrations, others skilled in the art can, by applying, current knowledge, readily modify and/or adapt the present invention for various applications without departing from the basic concept. Any modification and/or adaptations are intended to be within the meaning and scope of the appended claims of this invention.

A Simple and Rapid Non-Isotopic Microtiter Plate-Based Chromogenic Assay for Quantitative Detection of Abasic (AP) Sites in DNA.

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Key Words:
Abasic (AP) site, Oxidative DNA damage, Aldehyde Reactive Probe (ARP), DNA binding, Microtiter plate, Reactibind, Direct assay, Cost-effective.

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Abstract

Several methods for quantifying AP sites have been reported, allowing discrimination of low AP sites in DNA. However, some of these methods require radioactivity. Others not requiring radioactivity are either extremely time consuming, technique-sensitive, or cumbersome. We developed a rapid, simple, sensitive and cost-effective method to detect and quantify abasic sites in DNA by reacting an aldehyde group-specific biotinylated chemical reagent called Aldehyde Reactive Probe (ARP) with AP sites in DNA directly on the microtiter plate. This unique method obviates the need for ethanol precipitation or ultrafiltration to remove excess chemical probe from DNA before adding to plate, in variance to previous ARP methods. The biotin-tagged AP site was detected and the number determined colorimetrically using avidin-biotin-horseradish-peroxidase conjugate method. We used Reacti-bind DNA coating solution (Pierce Chemical, Rockford, Illinois, and U.S.A.) to immobilize DNA to the microtiter plate. An enhanced and consistent binding of DNA to microtiter plate was achieved. The assay was able to determine accurately, specific AP sites in calf thymus DNA generated by acid/heat depurination method, and spontaneously generated AP sites by incubation at physiological conditions (pH 7 and 37 C). The new Direct ARP assay was sensitive enough to measure 0.5 AP/10^5 bp (1 AP site/5 x 10^6 bases) in 190 ng of double stranded DNA bound to plate. This current development is easy, rapid and inexpensive method to determine the number of AP sites in human genomic DNA, with possibility of automation for large number of samples. Moreover, when this method is N-glycosylase enzyme-coupled, it could be adapted to estimate DNA repair capacity in cells and tissues using the principle of enzymatic processing.

Introduction

The formation of abasic sites (AP sites) resulting from the removal of purine or pyrimidine bases is among the most common oxidative lesions in DNA. Endogenous and exogenous processes can give rise to abasic sites. In humans, it was estimated that about 10 000 AP sites are produced per cell every day by hydrolysis of the N-glycosylic bond under physiological conditions (1). Ionizing radiation (2), potent carcinogens, chemical agents such as bleomycin (3) and alkylating agents (4) also promote the formation of AP sites. In addition, AP sites are intermediates in the base excision repair pathway, where damaged base is being removed by DNA N-glycosylases as the first step in base excision repair process (5). Deficiency in the repair pathway and increasing oxidative stress could contribute to increased background levels of AP sites. Since AP site lesion was shown to be strong block to DNA synthesis in vitro (6), AP sites accumulation could cause cell death and/or mutation induction (7,8). Accumulating evidence from recent studies implicate increased background level of oxidative DNA base damages in the pathogenesis of some human diseases. These include Alzheimer's disease (9), amyotrophic lateral sclerosis (10), Parkinson's disease (11), cataract formation, aging process (12) and some types of cancers (13,14). Cells and human tissues are also being screened for specific DNA damage in order to correlate the action of toxic agents with human diseases (15,16,17,18).

The biological significance of AP site enumerated, stimulate interests in developing specific and sensitive methods to detect and quantify abasic sites in DNA. Although several methods for measuring AP sites have been reported, many, when sensitive enough to measure low-level AP sites require radioactivity (19). Others, while not requiring radioactivity are either not sensitive (20), or do require costly equipment, skill-sensitive and time-consuming (21,22). A previous method attempting a solution to these challenges used Aldehyde Reactive Probe (ARP) to tag biotin to the aldehyde group of AP sites in DNA (23). The ELISA-like method reported promised to be of advantage in its specificity, but was limited in its sensitivity. Attributable to this limitation was two major problems. First, there is limitation of DNA binding to the UV-irradiated microtiter plate. Second, there is non-specific binding of ARP to the UV-irradiated microtiter plate. These cause inconsistent measurement and high background noise respectively, reducing the reliability of measurements. Another draw back was the laborious, but important step of ethanol precipitation to remove excess ARP that limits the use of the assay when available DNA sample is small. The current development allows rapid and sensitive measurement of AP sites in DNA directly on the microtiter plate, obviating the need for ethanol precipitation or ultrafiltration/centrifugation. We describe the development of a new non-isotopic microtiter plate-based chromogenic method to detect ARP-tagged aldehyde-containing AP sites in DNA. Subsequently, we demonstrate the application of the method to detect and measure AP sites in calf thymus DNA generated by heat/acid-buffer depurination (21), and spontaneously by incubation under physiological conditions (1,22).

Materials and Methods

Heat/Acid-Buffer Depurination of Calf thymus DNA.
We purchased pure grade double stranded calf thymus DNA from Sigma Chemical Co. Specific number of AP sites were selectively produced in the DNA by heat/acid-buffer treatment as

previously reported (21). On another hand, prior to the heat/acid-buffer treatment, the DNA was treated with 5 mM methoxyamine for 1 h at room temperature in order to remove traces of existing aldehyde. The methoxyamine was then removed by ethanol precipitation and the sample re-suspended in sodium phosphate buffer, pH 7. The methoxyamine-treated and methoxyamine non-treated DNA (100 ug/mL) were then dialyzed separately in 10 mM NaH_2PO_4, 100 mM NaCl and 10 mM sodium citrate at pH 5.0 (AP-buffer). The dialyzed DNA was heated at 70 C for 50 minutes and the reaction stopped by chilling rapidly on ice to create 5 AP sites/10^4 bp (20). Each sample was dialyzed back to pH 7.5 in PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H2O$, and 1.4 mM KH_2PO_4). This was appropriately diluted with control DNA to produce 1, 2, 3 and 4 AP sites/10^4 bp respectively. Appropriate DNA concentrations were also obtained by diluting with PBS buffer. To create relatively low-level AP site in methoxyamine treated DNA, 1 AP site DNA sample was appropriately diluted with control DNA to produce 0.1, 0.2, 0.4 and 0.8 AP site/10^4 bp respectively. Similarly, low-level AP site in non-treated DNA was created by appropriately diluting 2 AP-DNA sample with control DNA, to produce 0.2, 0.4, 0.8 and 1.6 AP site/10^4 bp respectively.

Immobilization of DNA to Microtiter Plate

In order to enhance binding of DNA to 96 well U-bottom high binding plate (Costar #3791-Costar corporation, Cambridge, MA), 200 uL of DNA at 1.25, 2.5, 5 and 10 ug/mL concentration were modified respectively with 300 uL Reacti-bind DNA coating solution (Pierce Chemical Corporation, Rockford, Illinois), resulting in 0.5, 1, 2, and 4 ug/mL DNA respectively after Reactibind modification. 100 uL of each mixture was added to each well respectively. This implies that, 50, 100, 200 and 400 ng of DNA respectively were introduced to the plate. The plate was incubated at room temperature overnight for 16 h. Unbound DNA was removed and plate was then washed three times with 0.1% Tween-PBS buffer (Phosphate buffered solution containing 0.1% Tween 20). Subsequent steps of the assay do not detach bound DNA nor alter the bound DNA.

The New Direct ARP Assay

In order to trap the open chain aldehyde generated in DNA at the position of AP sites, 100 uL of 1 mM of ARP was added to each well, and plate was incubated at room temperature for 1 h. After discarding contents, excess ARP in each well was removed by washing plate three times with 1% Tween-PBS buffer (Phosphate buffered solution containing 1% Tween 20), followed by washing with 0.1 % T-PBS once. The plate was swiped and dried without desiccating. 80 uL of 1:20 diluted ABC solution was then added to each well. The plate was covered with parafilm and incubated at 37 C for 1 h and then washed with 0.1 %Tween-PBS buffer three times. When Azinobis(3-ethylbenzo thiazoline-6-sulfonic acid) (ABTS) was used as substrate for horseradish peroxidase (HRP), 120 uL of the substrate prepared according to manufacturer's instruction was added into each well. After incubation at room temperature for 1 h, the absorbance was measured at 405 nm. When 3,3,5,5-Tetramethylbenzidine (TMB) (Moss Inc., Pasadena, Maryland, U.S.A.) was used as substrate for horseradish peroxidase, 160 uL of the substrate solution was added to each well and incubated at 37 C for 30 minutes. The absorbance was then measured at 650 nm. In all ARP assays, signals were expressed as the change in absorbance after subtracting background readings for control DNA. All experimental samples were in triplicates or more, with standard deviation less than 10 %.

Spontaneous Physiological Depurination of Calf Thymus DNA

AP sites were slowly generated in methoxyamine-treated calf thymus DNA by spontaneous depurination under physiological condition of pH 7.0 and incubation at 37 C for 4, 6, 8 and 10 days respectively. The number of abasic sites in the samples was then monitored using the present assay, the new direct ARP assay.

Results

Binding Efficiency of DNA to Plate

The amount of DNA bound to plate when DNA was immobilized using reacti-bind solution was determined by an ultrasensitive DNA quantitative fluorescent assay (PicoGreen) following the manufacturer's instructions. We determined the DNA concentration in each well after allowing binding overnight for 16 h. The amount bound was calculated from the difference before and after incubation, taking note of any change in volume after incubation. More than 90 % of DNA is bound when modified with Reactibind. The amount of DNA bound increased with time, while an optimum binding was ensured by 16 h. Figure 1 shows the amount of DNA bound to plate when DNA was modified by Reacti-bind DNA coating solution. There is a linear relationship between DNA bound and DNA concentration. When 5 and 10 ug/mL DNA were incubated for 16 h, 200 ng and 400 ng of DNA were in each wee respectively, and about 190 ng and 360 ng of DNA were bound to each well respectively. This indicates a binding efficiency greater than 90% was achieved.

AP Site determined by the New Direct ARP Assay

Figure 2 shows the ARP signal for selectively created AP sites in methoxyamine non-treated DNA at specific concentrations determined by the new direct ARP assay method using ABTS as horseradish peroxidase substrate. ARP signal increased with increasing AP sites in DNA. There is a linear relationship between ARP signal and DNA concentration. The sensitivity is higher for 10 ug/mL DNA than 5 ug/mL DNA (Table 1). At 10 ug/mL, when about 360 ng of DNA was bound in each well, the direct assay method was able to measure as low as 0.25 AP sites per 10^4 bp. At 5 ug/mL, when 200 ng DNA was started with and about 190 ng of DNA was bound in each well, the direct assay was able to measure as low as 0.4 AP site/10^4 bp.

Optimization of the New Direct ARP Assay for Low-level AP sites

Since obtaining 10 ug/mL of DNA from biological samples may prove difficult, requiring a large tissue mass source which may not be available, there is need to optimize the direct assay for smaller DNA concentrations. Moreover, higher sensitivity is required for determining low-level AP sites in DNA. In order to increase the sensitivity of the direct assay method, we used 3,3,5,5-Tetramethylbenzidine (TMB) (Moss Inc., Pasadena, Maryland, U.S.A.) in place of ABTS as horseradish peroxidase substrate. Figures 3A and 3B show the ARP signal for selectively created AP sites in methoxyamine non-treated DNA and methoxyamine-treated DNA respectively at specific concentrations, determined by the new direct ARP assay method. ARP signal increased with increasing AP sites in both treated and non-treated DNA. Also, there is a linear relationship between ARP signal and DNA concentration in both. The sensitivity is higher for 10 ug/mL DNA than 5 ug/mL DNA. The sensitivity increased with increasing DNA concentration and exceeds twice that of ABTS substrate (Table 1). Taken together, by varying DNA concentrations and horseradish peroxidase substrate, the new direct ARP assay can quantify very low AP sites in DNA.

Sensitive Detection of AP sites in DNA by the New Direct ARP Assay

To estimate the lowest number of AP sites detectable by the new direct ARP assay, we performed the assay under optimum conditions, varying the substrate and DNA concentrations to determine low-level AP sites. Figure 4A shows the ARP signal for 0, 0.2, 0.4, 0.8 and 1.6 AP site/10^4 bp respectively in 5 ug/mL of methoxyamine non-treated DNA. Figure 4B shows ARP signal for 0, 0.1, 0.2, 0.4, 0.8 and 1 AP site/10^4 bp respectively in 5 and 10 ug/mL of methoxyamine-treated DNA. This discrimination indicates that as low as 0.5-1 AP sites/10^5 bp in DNA could be measured by the new direct ARP assay method when DNA was pre-treated with methoxyamine to remove traces of pre-existing AP sites. Figure 5 shows the ARP signal as a function of time when methoxyamine-treated calf thymus DNA was kept in pH 7 phosphate buffer at 37 C (spontaneous physiological depurination), and assayed using the new direct method. The increase in AP sites was proportional to the incubation time. A linear increase in spontaneously generated AP sites was detected. Methoxyamine-treated DNA kept at 4 C under similar conditions did not show appreciable increase in ARP signal with time. These data indicate that approximately 1.56 AP sites/10^6 bp was introduced per day.

Discussion

We have shown that a new non-isotopic microtiter plate-based assay method using a biotinylated aldehyde group specific chemical reagent, aldehyde reactive probe (ARP) can quantitatively detect as low as 0.5 AP sites/10^5 pb (1 AP site/5 x 10^6 bases) in calf thymus DNA generated by heat/acid-buffer depurination. Further, the new assay was applied to monitor spontaneous depurination under physiological conditions up to 9 days. A previous method using ARP to quantify the aldehyde containing AP sites in calf thymus DNA was limited in its sensitivity, being able to quantify only 10 AP site/10^5 bp (23). This limitation was ascribed to non-specific sites contributing to high background signal, and low binding of DNA to the UV irradiated plate used. Recently, an improvement on this method was reported using protamine sulfate coated microtiter plate and ethanol precipitation to remove excess ARP. Nevertheless, it could only measure 0.5 AP sites/10^4 bp (1 AP site/5 x 10^5) (24). While ARP was promising in its specificity, appropriate method for optimum sensitivity and feasibility was not achieved using the simplicity of the microtiter plate (ELISA-like method) in these attempts. Among the important criteria to consider when assessing the usefulness of an assay are its specificity, sensitivity, and simplicity. These challenges have been met in the current development.

The new direct ARP assay method is rapid, simple and more sensitive than previously reported ARP assay methods. More importantly, it eliminates the laborious and time consuming ethanol precipitation or ultrafiltration/centrifugation step that limited the earlier methods when available DNA sample was small. In the current method, DNA was efficiently immobilized directly to the plate when modified by reacti-bind solution without introducing non-specific sites. Starting with as small as 200 ng DNA, 190 ng was bound using 5 ug/mL DNA in the new direct assay method, compared to 70-ng bound using 10 ug/mL DNA with UV irradiated plate (23). The new direct ARP assay then measured as low as 0.5 AP sites/10^5 bp (1 AP site/5 x 10^6 base). By using the new direct ARP assay method to quantify spontaneously generated AP sites in double stranded calf thymus DNA under physiological conditions, we derived that approximately 1.56 AP sites/10^6bp/day are generated (Fig.5). This translates to about 9 360 AP sites generated /cell/day in the mammalian genome. This is in agreement with two recent reports monitoring spontaneously generated AP sites

in double stranded calf thymus DNA. Nakamura et al. (1998) measured 1.54 AP sites/10^6 bp/day translating to about 9 000 AP sites/cell/day (17) and Maulik et al. (1999) measured 1.7 APsites/10^6bp/day translating to about 10 200 AP sites /cell/day (22). Previously, 10 000 AP sites/cell/day was estimated using the depurination rate of DNA at 70 C and physical chemistry (1).

Advantages of the New Direct ARP Assay

As demonstrated, the new direct ARP assay method is specific and sensitive to detect and quantify AP sites in genomic DNA as low as 0.5 AP site/10^6 bp (1 AP site/5 x 10^6bases). Although new systems that can monitor lower AP sites are emerging (23,24), it is clear that the procedures involved is either not so simple as in the current development or are costly and skill-sensitive. There are other advantages of the current method over other methods for quantifying AP sites. The new Direct ARP assay obviates the need for the required laborious ethanol precipitation to remove excess ARP, requiring extremely small amount of starting DNA (200-500 ng) when compared with other methods (23,24). The procedure in this current development is directly on the microtiter plate from the beginning of the assay to the end. Therefore, this current technology indicates the possibility for automating quantitative detection of abasic site lesion in DNA. We are presently measuring the amount of endogenous AP sites using the new direct ARP assay in genomic DNA from central and peripheral tissues of normal and neurodegenerative disorders. In addition to this anticipated potential clinical application, the new direct ARP assay method when N-glycosylase enzyme-coupled, may be adapted to estimate DNA repair capacity in cells and tissues.

In conclusion, we developed a new direct assay to determine AP site in DNA by reacting ARP with DNA directly on the microtiter plate. This easy, rapid and cost-effective method obviates the need for ethanol precipitation, while sensitive enough to determine as low as 5 AP site/10^6 bp.

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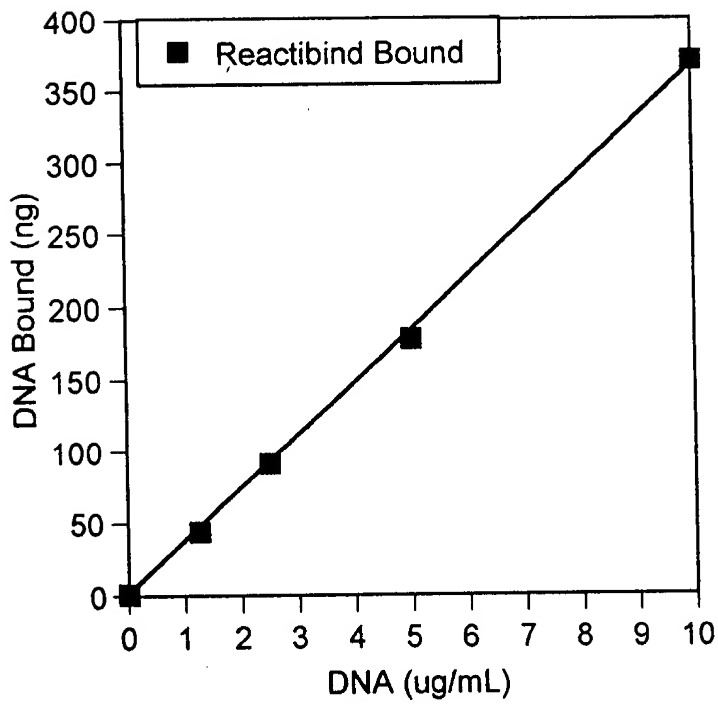

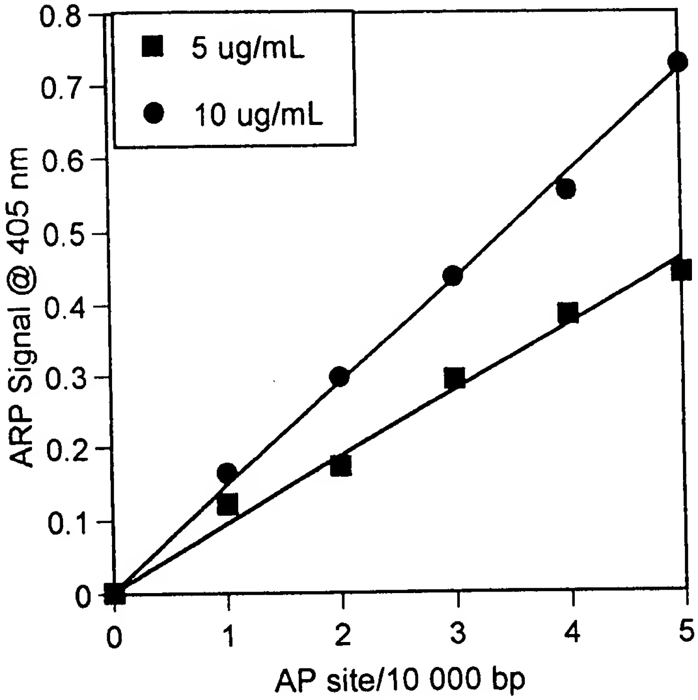

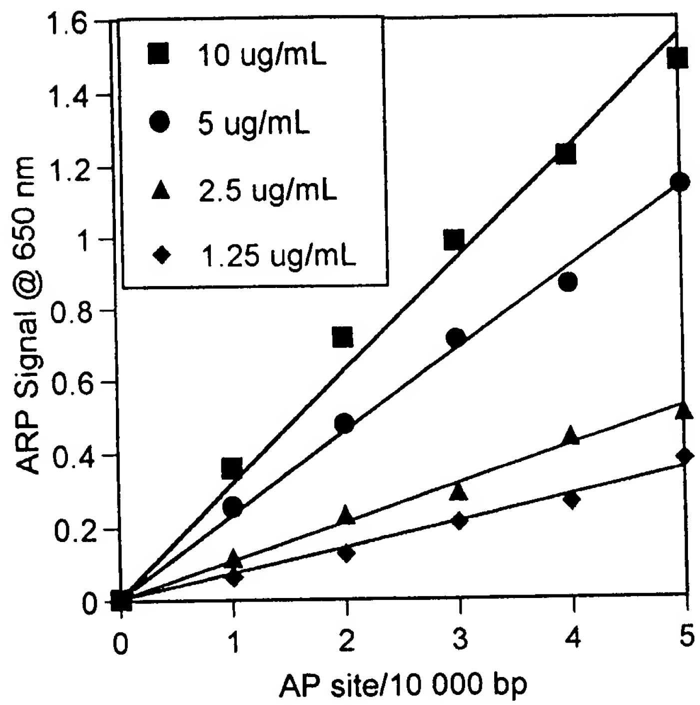

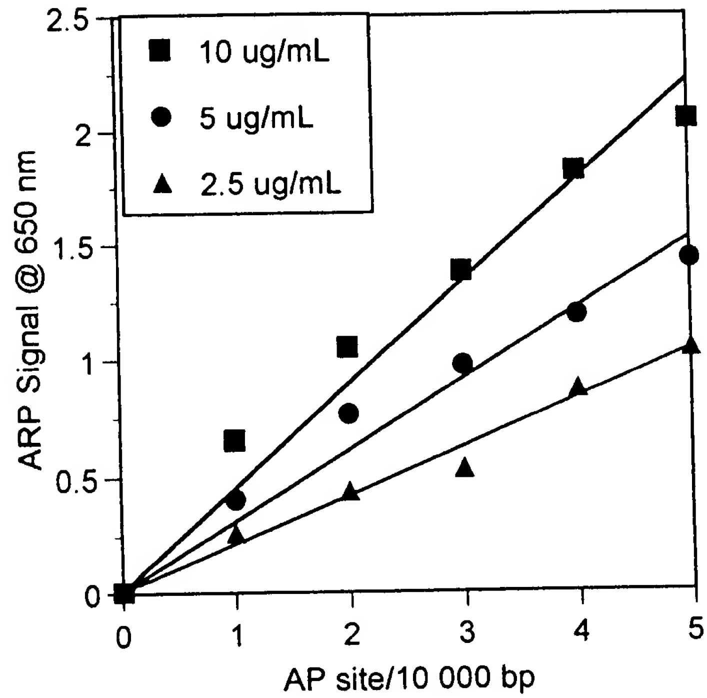

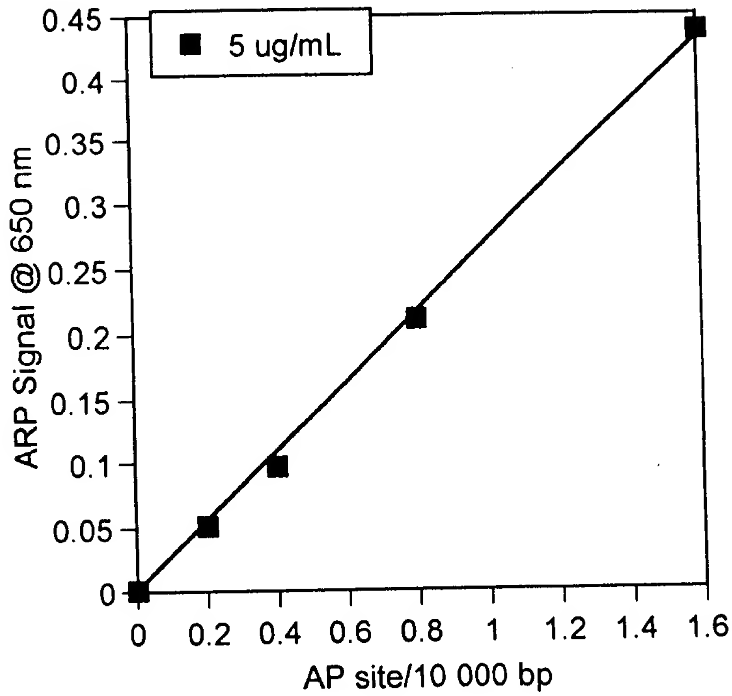

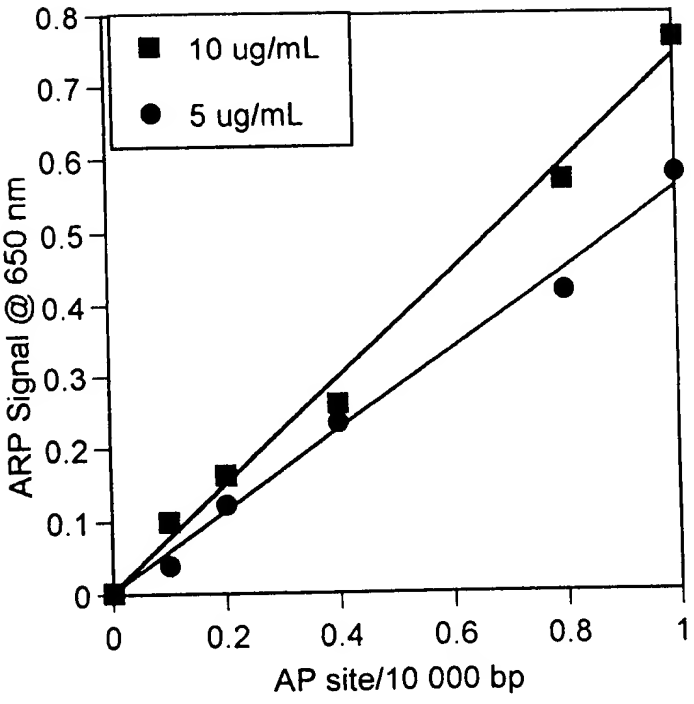

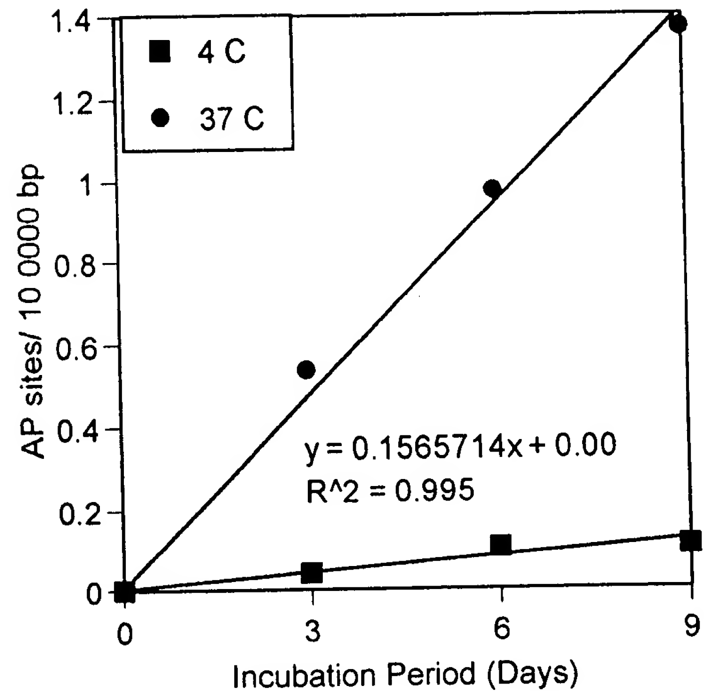

	Slope x 10^{-3} (OD/min)	Slope x 10^{-3} (OD/min)	Slope x 10^{-3} (OD/min)
DNA (ug/mL)	**Direct Assay (ABTS)**	**Direct Assay (TMB). Methoxyamine**	
		Non-treated	**Treated**
1.25	-	7.00	-
2.5	-	10.40	-
5.0	8.58	22.60	55.54
10.0	12.60	30.90	73.55
Lowest AP Quantified	0.3 AP site/10 000 bp	0.1 AP site/10 000 bp	0.05 AP site/10 000 bp

Table 1: Comparing the Sensitivity of Direct ARP Assay Methods

Legend to Figures

Figure 1. Fluorometric determination of Reacti-bind modified DNA binding efficiency to plates. 200 uL of DNA at the indicated concentration was mixed with 300 uL of reacti-bind DNA coating solution. 100 uL of the 2:3 mixture was added to each well of "High Binding" Costar plates. After incubating at room temperature overnight for 16 h, the DNA concentration in each well was determined by an ultrasensitive fluorometric method (PicoGreen) following the manufacturer's instructions. The amount of DNA bound to each well was then computed as described in the "Materials and Methods" section. Each point represents the mean of three samples.

Figure 2. ARP signal of DNA containing specific AP site/10 000 bp determined by the direct assay method using ABTS substrate. 200 uL of calf thymus DNA at the indicated concentration was mixed with 300 uL of reacti-bind DNA coating solution. 100 uL of the mixture was added to each well of "High binding" Costar plates. Subsequent steps for the direct assay method were performed as described in the "Materials and Methods" section. Each point represents the mean of three samples. The ARP signal for 5ug/mL and 10 ug/mL DNA are expressed as the change in absorbance at 405 nm after subtracting background readings obtained for control DNA.

Figure 3a. ARP signal of calf thymus DNA containing specific AP site/10 000 bp determined by the direct assay method using TMB substrate. 200 uL of calf thymus DNA at the indicated concentration was mixed with 300 uL of reacti-bind DNA coating solution. 100 uL of the mixture was added to each well of "High binding" Costar plates. Subsequent steps for the direct assay method were performed as described in the "Materials and Methods" section. Each point represents the mean of three samples. The ARP signals are expressed as the change in absorbance at 650 nm after subtracting background readings obtained for control DNA.

Figure 3b. ARP signal of Methoxyamine pretreated calf thymus DNA containing specific AP site/10 000 bp determined by the direct assay method using TMB substrate. 200 uL of calf thymus DNA at the indicated concentration was mixed with 300 uL of reacti-bind DNA coating solution. 100 uL of the mixture was added to each well of "High binding" Costar plates. Subsequent steps for the direct assay method were performed as described in the "Materials and Methods" section. Each point represents the mean of three samples. The ARP signal for 5ug/mL and 10 ug/mL DNA are expressed as the change in absorbance at 650 nm after subtracting background readings obtained for control DNA.

Figure 4a. ARP signal of calf thymus DNA containing specific Low AP site/10 000 bp determined by the Direct assay method using TMB substrate. 200 uL of 5 ug/mL calf thymus DNA containing indicated specific AP site was mixed with 300 uL of reacti-bind DNA coating solution. 100 uL of the mixture was added to each well of "High binding" Costar plates. Subsequent steps for the direct assay method were performed as described in the "Materials and Methods" section. Each point represents the mean of three samples. The ARP signals are expressed as the change in absorbance at 650 nm after subtracting background readings obtained for control DNA.

Figure 4b. ARP signal of Methoxyamine-treated calf thymus DNA containing specific Low AP site/10 000 bp determined by the direct assay method using TMB substrate. 200 uL of 5 ug/mL

Methoxyamine-treated calf thymus DNA containing indicated specific AP site was mixed with 300 uL of Reacti-bind DNA coating solution. 100 uL of the mixture was added to each well of "High binding" Costar plates. Subsequent steps for the direct assay method were performed as described in the "Materials and Methods" section. Each point represents the mean of three samples. The ARP signals are expressed as the change in absorbance at 650 nm after subtracting background readings obtained for control DNA

Figure 5. ARP signal of Methoxyamine-treated calf thymus DNA incubated at physiological pH 7 and 37 C, undergoing spontaneous depurination at 0, 3, 6 and 9 days respectively; compared to Methoxyamine-treated calf thymus DNA incubated at pH 7 and 4 C incubated for 0, 3, 6, and 9 days.

Table 1. Comparing the Sensitivity of Direct ARP Assay Methods. Table shows the sensitivity of the various direct ARP assay methods described in the "Materials and Methods" section.

Quantitative Detection of Abasic (AP) Sites in DNA and Analysis of DNA Base Modification and Repair by an Improved Aldehyde Reactive Probe (ARP) Assay.

Running Title: Quantitative Analysis of Oxidative DNA Damage and Repair

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Abstract

We describe an improvement to a previously reported method for detecting and quantifying abasic (AP) sites in DNA by aldehyde reactive probe (Kubo et al., 1992). Serious problems were identified using UV-irradiated plates that limited the sensitivity of the previous assay to 1 AP site per 10 000 bp. The current development improved the sensitivity of this assay up to five fold. In addition, when enzyme-coupled, we quantitatively detected endonuclease-III sensitive sites in DNA base modifications. We used 0.1% protamine sulfate coated plate to enhance DNA binding. Consequently, a tremendous improvement in the sensitivity of the assay was achieved, measuring as low as 0.24 AP sites per 10 000 bp. Alternatively, by using Reacti-bind DNA coating solution (Pierce Chemical, Rockford, Illinois, and U.S.A.) to immobilize DNA to the microtiter plate, the sensitivity of the indirect ARP assay method measured as low as 0.1-0.2 AP sites per 10 000 bp. In both cases, enhanced and consistent binding of DNA to microtiter plate was achieved. The assay accurately determined specific AP sites introduced to calf thymus DNA by acid/heat depurination method. When we processed DNA base modifications using excess endonuclease III, we successfully applied the new assay to quantify total endonuclease-III sensitive sites in the modified DNA. The current improvement produced a far more sensitive and cost-effective method to determine the number of AP sites in human genomic DNA. When enzyme-coupled, the new assay can quantitatively detect specific base damages in DNA, and indirectly estimate specific DNA N-glycosylase enzyme repair capacity. The assay has a great potential for both clinical and research application.

Introduction

The formation of abasic (AP) sites resulting from the removal of purine or pyrimidine bases is among the most common oxidative lesions in DNA. Endogenous and exogenous processes can give rise to abasic sites. Ionizing radiation (von Sonntag et al., 1987), potent carcinogens, chemical agents such as bleomycin (Rabow et. al., 1986) and alkylating agents (Loeb and Preston, 1986) also promote the formation of AP sites. In addition, AP sites are intermediates in the base excision repair (BER) pathway. DNA N-glycosylases hydrolyze the N-glycosylic bond between the target base and deoxyribose, thus releasing a free base and leaving an apurinic/apyrimidinic (AP) site in DNA (Barnes et al., Wang et al., 1997). Deficiency in the repair pathway and increasing oxidative stress could contribute to increased background levels of AP sites. AP sites accumulation could cause cell death and/or mutation induction (Kamiya, et. al., 1992; Kunkel, 1984). Accumulating evidence from recent studies implicate increased background level of oxidative DNA base damages in the pathogenesis of some human diseases, including Alzheimer's disease (Mecoci et al., 1998), amyotrophic lateral sclerosis (Olkowski, 1998), Parkinson's disease (Mecocci et al., 1994), cataract formation, aging process (Mecocci et al., 1999) and some types of cancers (13,14). Therefore, cells and human tissues are being screened for specific DNA damage in order to correlate the action of toxic agents with human diseases (Nakamura et al., 1998; Stone et al., 1995; Baan et al., 1988; Mori and Dizdaroglu, 1993). Most recently, Collins et al.(1998), reported the usefulness of DNA damage in lymphocytes as a marker of oxidative stress in diabetes, correlating closely with clinical markers of diabetes. They noted in particular that high levels of formamidopyrimidine glycosylase-sensitive sites seem to represent changes specifically related to hyperglycemia. Ionizing radiation produces a plethora of base and sugar damages that can be cytotoxic or mutagenic (Wallace, 1998). Among the most common DNA base modifications occurring after ionizing radiation exposure is 5,6-dihydroxyhydrothymine (thymine glycol) (Leadon, 199) and its biological consequence has been well studied (Wallace , 199). Its quantitative detection is gaining acceptance as possible indicator of DNA damage and repair capacity after ionizing radiation exposure. When completely processed by its specific repair enzyme, endonuclease III, the resulting intermediate AP site could be quantified.

The biological significance of AP site enumerated, stimulate interests in developing specific and sensitive methods to detect and quantify abasic sites in DNA. Although several methods for measuring AP sites have been reported, many, when sensitive enough to measure low-level AP sites require radioactivity (Weinfeld et al., 1990). Others, while not requiring radioactivity are either not sensitive (Chen et al., 1992), or do require costly equipment, skill-sensitive and time-consuming (Nakamura et al., 1998; Maulik et al., 1999). A previous method attempting a solution to these challenges used Aldehyde Reactive Probe (ARP) to tag biotin to the aldehyde group of AP sites in DNA (Kubo et al., 1992). The ELISA-like method reported promised to be of advantage in its specificity, but was limited in its sensitivity. Attributable to this limitation was two major problems. First, there is limitation of DNA binding to the UV-irradiated microtiter plate. Second, there is non-specific binding of ARP to the UV-irradiated microtiter plate. These cause inconsistent measurement and high background noise respectively, reducing the reliability of measurements. We report two methods that improved the sensitivity of the ARP assay up to five times. First, by using 0.1% protamine sulfate coated plate to enhance DNA binding, a tremendous improvement in the sensitivity of the indirect ARP assay was achieved. Subsequently, we demonstrate the

application of the assay method to detect and measure specific AP sites introduced into calf thymus DNA by heat/acid-buffer depurination (Nakamura et al., 1998). Again, when we processed chemically produced DNA modification using endonuclease III, we accurately and specifically quantified endonuclease III-sensitive sites to demonstrate further the usefulness of the newly improved ARP assay.

Materials and Methods

Creation of Specific AP in Calf thymus DNA by Heat/Acid-Buffer Depurination.

We purchased pure grade double stranded calf thymus DNA from Sigma Chemical Co. Specific number of AP sites were selectively produced in the DNA by heat/acid-buffer treatment as previously reported (Nakamura et al., 1998). On another hand, prior to the heat/acid-buffer treatment, the DNA was treated with 5 mM methoxyamine for 1 h at room temperature in order to remove traces of existing aldehyde. The methoxyamine was then removed by ethanol precipitation and the sample re-suspended in sodium phosphate buffer, pH 7. The methoxyamine-treated and methoxyamine non-treated DNA (100 ug/mL) were then dialyzed separately in 10 mM NaH_2PO_4, 100 mM NaCl and 10 mM sodium citrate at pH 5.0 (AP-buffer). The dialyzed DNA was heated at 70 C for 10, 20, 30, 40, and 50 minutes and the reaction stopped by chilling rapidly on ice to create 1, 2, 3, 4 and 5 AP sites/10^4 bp respectively (Ide et al., 1993). Each sample was dialyzed back to pH 7.5 in PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H2O$, and 1.4 mM KH_2PO_4).

ARP Reaction

ARP reaction with the created specific AP DNA was performed as previously published (Kubo et al., 1992; Ide et. al., 1993) with modifications. 100 ug/mL of calf thymus DNA containing specific AP sites was pre-treated with 5 mM ARP and incubated at room temperature for 1 h. DNA was precipitated with 0.3 M sodium acetate in 100 % ethanol and the supernatant removed by centrifuge after incubating at –20 C for 1 h. The recovered residue was washed twice in 70 % ethanol, dissolved in PBS buffer and dialyzed in 10 mM NaH_2PO_4 buffer at pH 7.5 to further remove any excess ARP. Appropriate DNA concentrations were obtained by diluting each sample with PBS buffer. The ARP pre-treated DNA was then added to microtiter plate for indirect ARP assay.

Immobilization of DNA to microtiter plate by 0.1 % protamine sulfate

Protamine sulphate-coated plates were prepared by adding 200uL of 0.1% protamine sulphate to each well of 96 well U-bottom "High Binding" plate (Costar #3791-Costar corporation, Cambridge, MA), and the plate was incubated at room temperature overnight. Plates were then washed three times with distilled water, dried completely and stored in the dark. 100 uL of 0.25, 0.5, 1.0, 1.5 and 2.0 ug/mL DNA was added to each well respectively. This implies that 25, 50, 100, 150 and 200 ng of DNA were added to each well. Plates were then incubated overnight at 4 C for 16 h. Unbound DNA was removed and plate was then washed five times with 0.1% Tween-PBS buffer (Phosphate buffered solution containing 0.1% Tween 20). Subsequent steps of the assay do not detach bound DNA nor alter the bound DNA.

Immobilization of DNA to microtiter plates by Reactibind DNA coating solution.

In order to enhance binding of DNA to 96 well U-bottom high binding plate (Costar #3791-Costar corporation, Cambridge, MA), 100 uL of ARP pre-treated DNA at 0.25, 0.5 and 1.0 ug/mL

concentration was added to each well and modified respectively with 100 uL Reacti-bind DNA coating solution (Pierce Chemical Corporation, Rockford, Illinois). This implies that, 25, 50 and 100 ng of DNA respectively were introduced to the plate. The plate was incubated at room temperature overnight for 16 h. Unbound DNA was removed and plate was then washed three times with 0.1% Tween-PBS buffer (Phosphate buffered solution containing 0.1% Tween 20). Subsequent steps of the assay do not detach bound DNA nor alter the bound DNA.

Improved Indirect ARP assay

In both methods after incubation, plates were washed 5 times with 0.1 % T-PBS (Phosphate buffer solution containing 0.1% Tween 20) and then dried without desiccating. 50 uL of 1:20 diluted ABC solution (avidin-biotinylated horseradish peroxidase complex by Vector Laboratories Inc., Burlingame, CA), was added into each well. The plate was covered with parafilm and incubated at 37 C for 1 h and then washed with T-PBS five times. Azinobis(3-ethylbenzothiazoline-6-sulfonic acid), diammonium salt (ABTS, purchased from Vector Laboratories Inc., Burlingame, CA), was used as substrate for horseradish peroxidase (HRP). 100 uL of the substrate, prepared according to manufacturer's instruction was added into each well. After 30-45 minutes, the absorbance was measured at 405 nm. In all ARP assays, signals were expressed as the change in absorbance after subtracting background readings for control DNA.

Enzyme-Coupled ARP Assay

5,6-dihydroxyhydrothymine base modification (thymine glycol) was produced in calf thymus DNA in a dose dependent manner following exposure to osmium tetroxide by the procedure of Kow and Wallace (1985). Essentially 100 ug/mL of calf thymus DNA was partially denatured by heating at 70 C for 5 minutes, reacted with appropriate amount of 0.4% stock OsO4 to give 0.32% OS)4-treated DNA. This was appropriately diluted with control DNA to give 0.08 and 0.04% samples. All samples were then dialyzed twice against 10 mM Tris-HcL. 1 mM EDTA, at pH 7.). The number of thymine glycols present in the DNA was previously determined (Hubbard et al., 1989). 0.01 % OsO_4 treatment gives 1 fmol of thymine glycol, such that 0.04, 0.08 and 0.16 % OsO_4 treatment gave 4, 8 and 16 fmol of thymine glycol respectively. Each sample was completely processed using E.coli endonuclease III, isolated and purified as previously described ( ) at 5uL/50 ug/mL DNA, and then reacted with ARP as described earlier. The resulting abasic sites after endonuclease III processing were tagged by the biotinylated ARP. The improved indirect ARP assay was then used to determine the ARP signal in each sample.

Results

Binding efficiency of DNA to microtiter plate

In order to determine the amount of DNA bound to plate when immobilized using protamine sulfate coating and reacti-bind coating solution respectively, we employed an ultrasensitive DNA quantitative fluorescent assay (PicoGreen) following the manufacturer's instructions. We determined the DNA concentration in each well after allowing binding overnight for 16 h. The amount bound was calculated from the difference in DNA concentration before and after incubation. Figure 1a shows the amount of DNA bound to protamine sulfate coated plate. There is a linear relationship between DNA bound and DNA concentration. A binding efficiency increasing with increasing DNA concentration up to a peak of approximately 50% was observed. Figure 1b shows the amount of DNA bound to plate when DNA was modified by Reacti-bind DNA coating

solution. There is a linear relationship between DNA bound and DNA concentration. A binding efficiency similar to that of protamine sulfate coated plates was observed at the DNA concentration tested.

Improved Indirect ARP assay

Figure 2 shows ARP signals for specific AP site at indicated DNA concentrations with protamine coated plates. Although the sensitivity increased with increasing DNA concentration, there seems to be a more consistent value with lower concentrations. This probably could be explained from the fact that there is a consistent binding of DNA with lower DNA concentrations as shown in Figure 1a. At 1 ug/mL DNA concentration, 51.8 ng of DNA was bound and the sensitivity of the indirect method was about 0.25 AP sites per 10,000 bp. Taken together, it seems that the optimum performance of this new indirect ARP method is at DNA concentrations between 0.25-1 ug/mL when protamine coated plates are used. When Reactibind was used to immobilize DNA to plate, Figure 3 shows the ARP signals for specific AP site at indicated DNA concentrations with ABTS as substrate.

Optimization of the Improved Indirect ARP Assay

To further increase the sensitivity of the improved indirect ARP assay method, we used 3,3,5,5-Tetramethylbenzidine (TMB) (Moss Inc., Pasadena, Maryland, U.S.A.) in place of ABTS as horseradish peroxidase substrate. 100 uL of TMB substrate was added into each well. After 30-45 minutes, the absorbance was measured at 650 nm. TMB was not compatible with protamine sulfate coated plates, but it was found useful in Reactibind-modified DNA. Figure 4 shows ARP signals for specific AP site at indicated DNA concentrations with TMB as substrate.

Sensitivity of the Improved Indirect ARP Assay for Detecting Low-level AP sites

To verify the sensitivity of the current assay in determining low-level AP sites in DNA, we created relatively low-level AP site in methoxyamine treated DNA by appropriately diluting 1 AP site DNA sample with control DNA to produce 0.1, 0.2, 0.4 and 0.8 AP site/10^4 bp respectively. Figure 5 shows the resulting ARP signal in methoxyamine-treated DNA at 2 ug/mL when Reactibind was used to immobilize DNA to plate. This indicates that the newly improved assay could measure as low as 0.1 AP sites in ARP pre-treated DNA (indirect ARP method).

Discussion

We improved upon a previously reported assay for quantitative detection of abasic sites in DNA. The improved assay using a biotinylated aldehyde group specific chemical reagent, aldehyde reactive probe (ARP) quantitatively detected as low as 1 AP sites/10^5 pb in calf thymus DNA generated by heat/acid-buffer depurination. This 10 fold increased sensitivity when compared to the previous assay reported by Kubo et al., (1992). The limitation of the previous assay in sensitivity was ascribed to non-specific sites contributing to high background signal, and low binding of DNA to the UV irradiated plate used. While ARP was promising in its specificity, appropriate method for optimum sensitivity and feasibility was not achieved using the simplicity of the microtiter plate (ELISA-like method) in these attempts. Among the important criteria to consider when assessing the usefulness of an assay are its specificity, sensitivity, and simplicity. These challenges have been met in the current development. A recent attempt to improve the previous assay was reported by Asaeda et al.(1998) using protamine sulfate coated microtiter plate as in the current development

after pre-treating calf thymus DNA with ARP. It measured as low as 0.5 AP sites/10^4 bp (5 AP site/ 10^5 bp) using ABTS as substrate. Eventhough similar amounts of DNA were bound to protamine sulfate coated plates in the two cases, we measured as low as 0.24 AP sites/104 bp, a two fold increased sensitivity using ABTS substrate. Possible explanations for the different sensitivities include the differences in ARP-pretreatment protocol and subsequent assay methods. More importantly, the current development alternatively immobilized DNA after modifying with Reactibind solution. This allowed the use of a compatible substrate, TMB that resulted in greater sensitivity than ABTS substrate. While Asaeda et al. (1998) suggested the use of *o*-phenylenediamine (*o*-PDA), a substrate compatible with protamine sulfate coated plates in increasing sensitivity to about 0.5 AP sites/105 bp (5 AP sites/106 bp), is carcinogenic. Its routine use is limited, therefore presenting another limitation of the ARP assay in their described method.

The improved ARP assay was able to quantitatively detect thymine glycol residues in calf thymus DNA when coupled to endonuclease III enzyme. After complete enzymatic processing of chemically created thymine glycol in calf thymus DNA, the resulting abasic sites were determined by the assay. We established that 1 TG would give ARP signals of approximately 0.3 OD at 405 nm. The assay can measure as low as 0.25 TG/10 000 bp (in calf thymus DNA), about 2 fmol of TG. Immunochemical method of quatitating thymine glycol using monoclonal antibody reported by Hubbard et al., 1989 and Chen et al., 1990 detected as low as 0.5-0.75 TG/10 000 bp. The sensitivity of the current development using enzyme coupled specific chemical reagent probe is doubled. Multiply damaged sites are produced by ionizing radiation as a result of the spatial ionization of water resulting in bursts of free radicals within the vicinity of DNA molecule. It is possible to process specific base modification in such a spectrum of damages using substrate specific base excision repair enzyme coupled to the current assay. Again, the activity of a specific DNA base excision repair in a biological sample can be estimated when compared to an optimally active enzyme.

Advantages of the New Direct ARP Assay

We are presently measuring the amount of endogenous AP sites using the new direct ARP assay in genomic DNA from central and peripheral tissues of normal and neurodegenerative disorders. In addition to this anticipated potential clinical application, the new direct ARP assay method when N-glycosylase enzyme-coupled, may be adapted to estimate DNA repair capacity in cells and tissues. In conclusion, we developed a new direct assay to determine AP site in DNA by reacting ARP with DNA directly on the microtiter plate. This easy, rapid and cost-effective method obviates the need for ethanol precipitation, while sensitive enough to determine as low as 5 AP site/10^6 bp.

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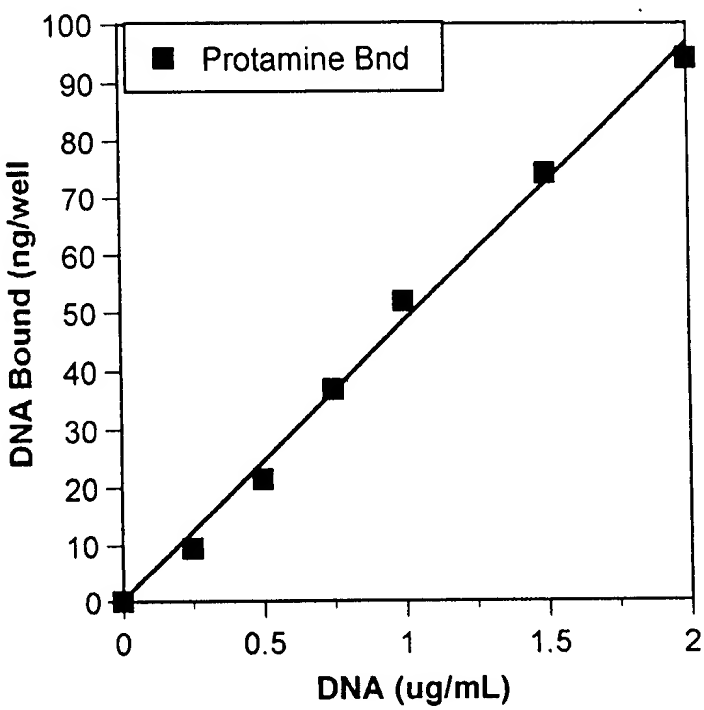
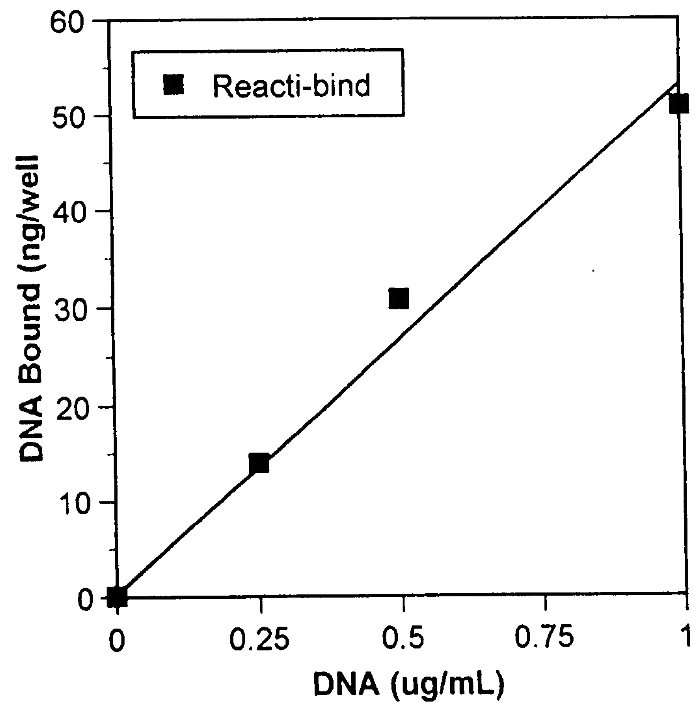
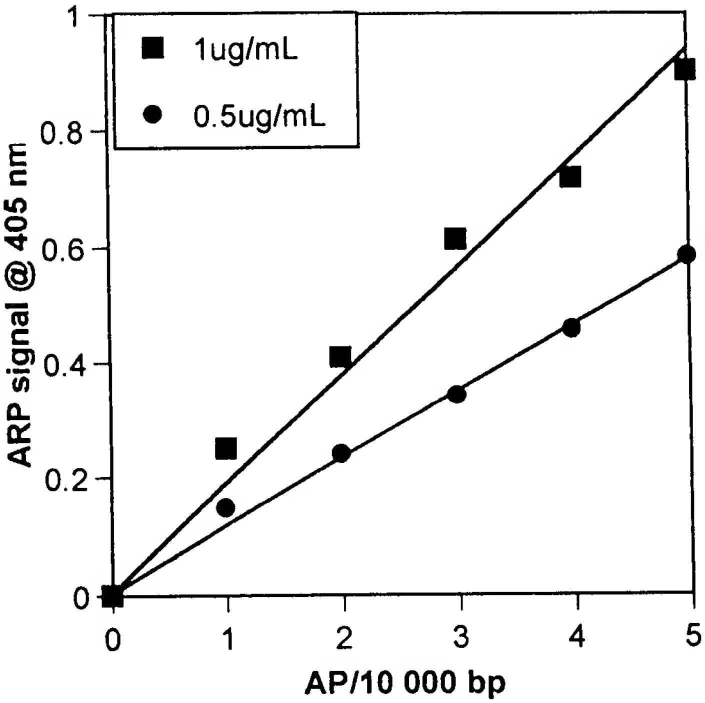
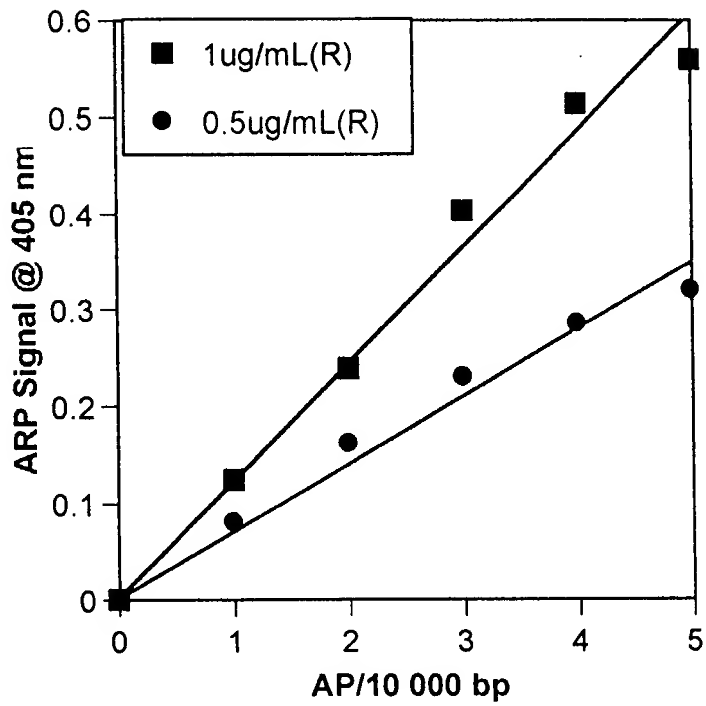
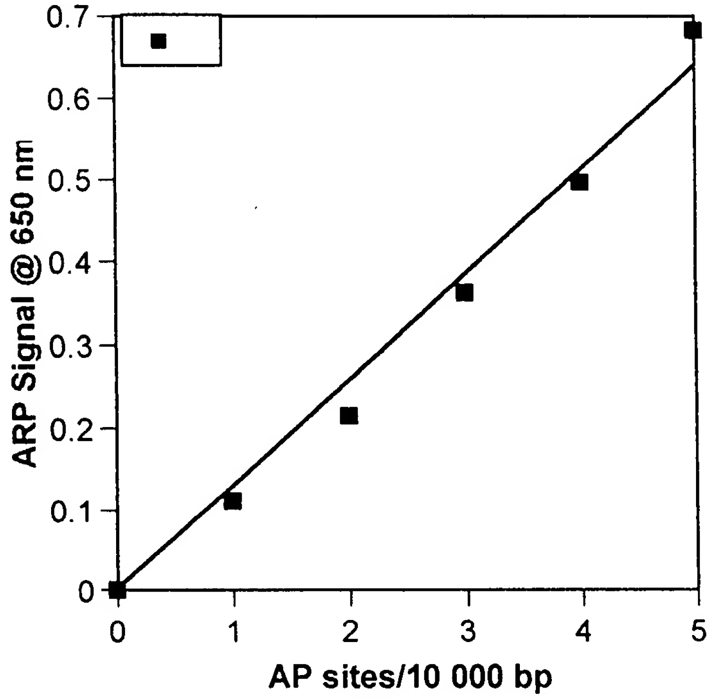
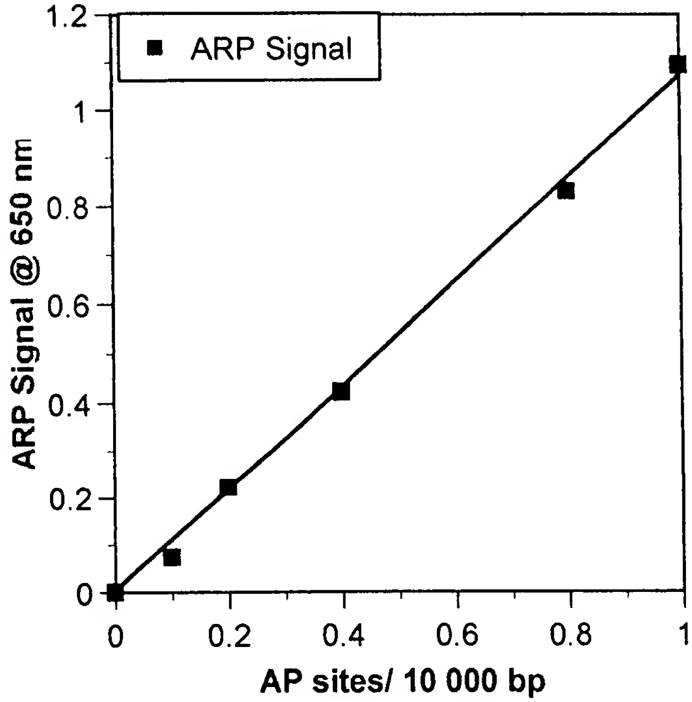


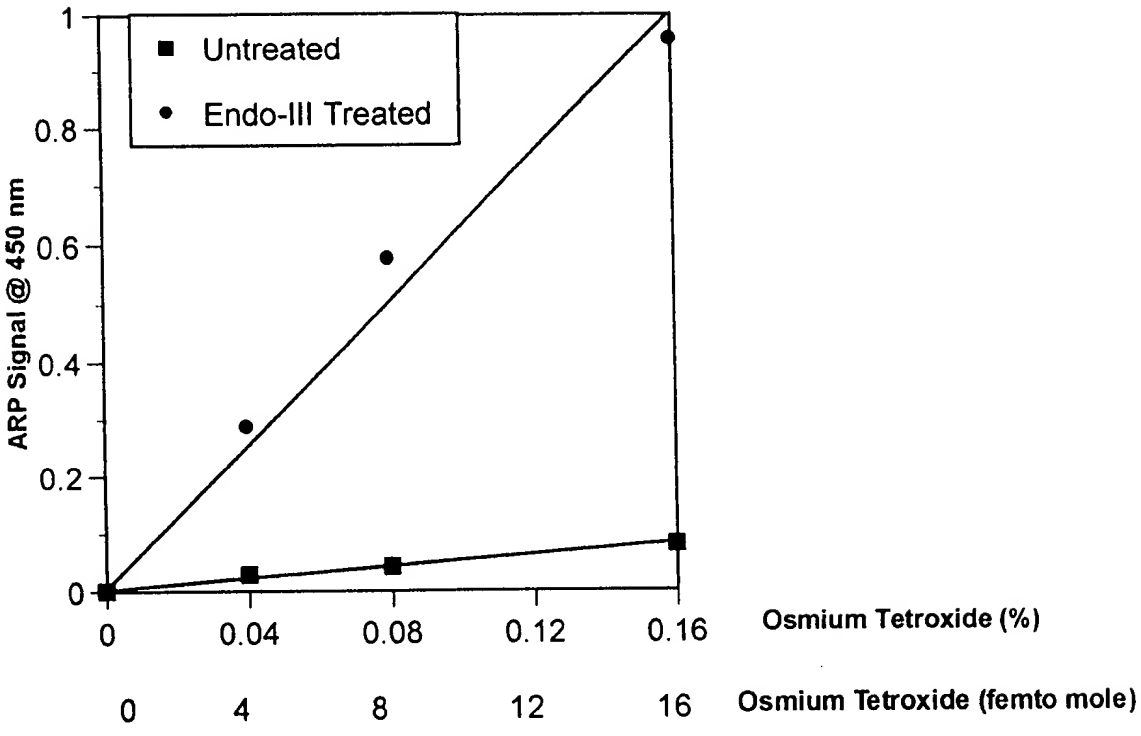

Reacti-bind
DNA Bound (ng/well)
DNA (ug/mL)
0
10
20
30
40
50
60
0
0.25
0.5
0.75
1

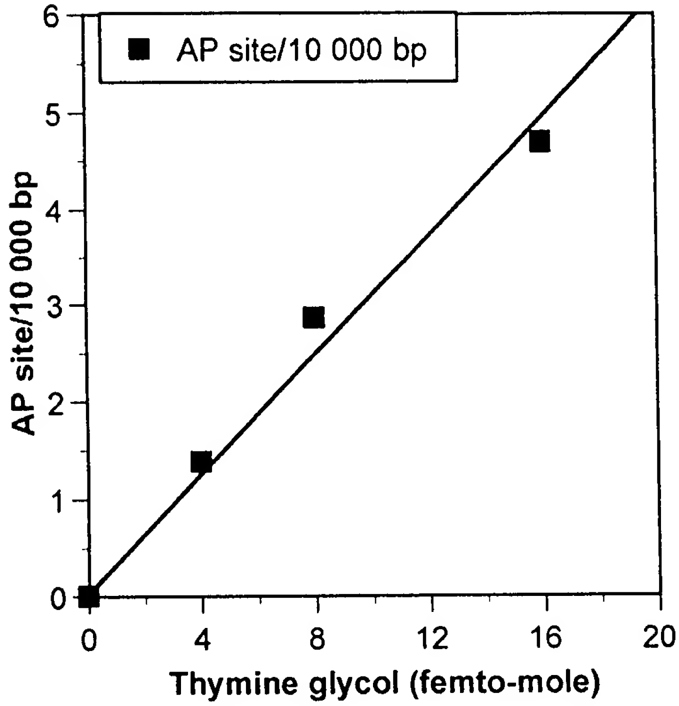










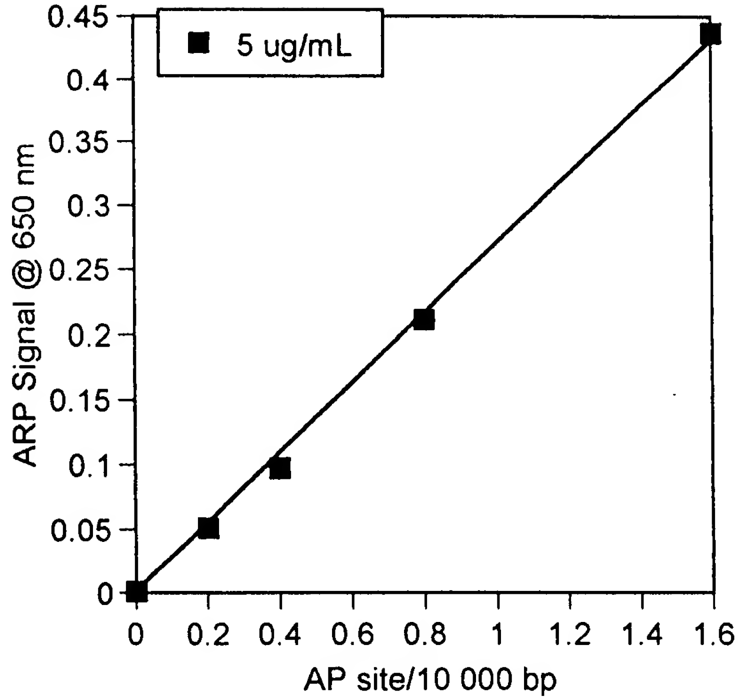


RAPID ASSAY FOR DNA DAMAGE AND REPAIR USING SILICON MICROSTRUCTURES.

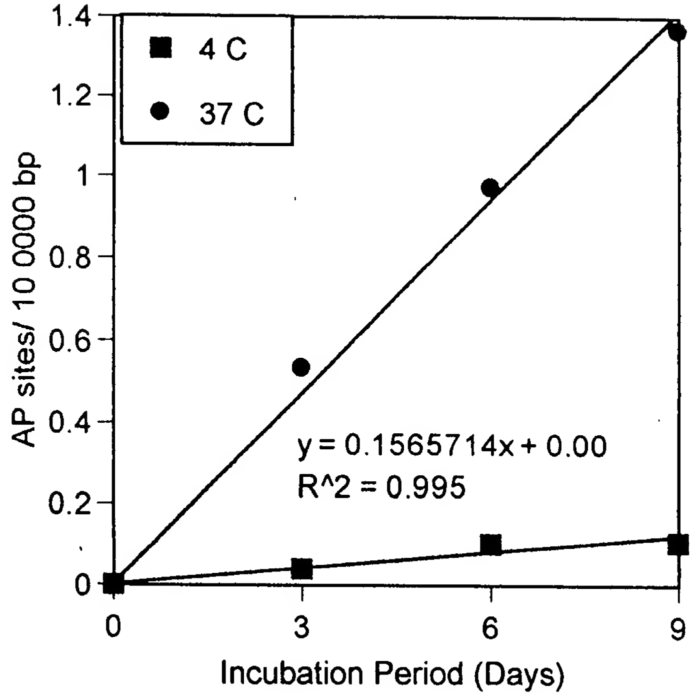
Abstract of Research Plan

Cells and human tissues screened for specific DNA damage could be used to correlate the action of clastogenic agents with human diseases and their rapid detection may be required in a number of important health-related areas. The ultimate goal of this multi-phase project is to commercialize the development of a portable DNA damage/repair quantitative detection system, easy to use by relatively unskilled personnel, yet providing a rapid-on-the spot screening result of tissue or blood cells for specific genomic DNA damage, and indicate susceptibility to a certain cancer or genetic disease. Among the DNA damage produced by oxidative stress commonly considered is abasic site (AP site) lesion and it is produced either endogenously, or exogenously by genotoxic agents, toxicological drugs and ionizing radiation. Our preliminary data demonstrate a chemical reagent probe specific for abasic sites consistently and efficiently measured approximately 0.5 AP sites/10^5 bp in acid/heat stressed double stranded calf thymus DNA directly on the microtiter plate. It is yet to be tested if similar efficiency could be obtained using recently developed silicon microstructures and application of microfluidics technologies for extracting DNA from complex biological samples towards automation. The **specific aim** of the current phase-I is to quantitatively detect accurate abasic sites in genomic DNA of normal and pathological/free radical-treated cells and tissues by combining the application of silicon microstructures and microfluidics technologies with the demonstrated chemical reaction and optical detection systems in a timely, efficient and cost effective manner.

Introduction: Rapid detection of specific DNA damage may be required in a number of important health-related areas. Genomic instability in cells is a compromise between DNA damage and proper DNA repair. Therefore, cells and human tissues are continually screened for specific DNA damage/DNA repair capacity that could be used to correlate the action of clastogenic agents with certain type of cancer or genetic disease. While abasic sites (AP sites) in DNA, 8-oxoguanine and thymine glycols are lesions of oxidative stress, among the DNA damage commonly considered is generation and measurement of Apurinic/apyrimidinic sites (AP sites) produced either endogenously, or exogenously by genotoxic agents, toxicological drugs and ionizing radiation. Recent reports indicate that abasic sites can induce triplet-repeat expansions (TRE) during replication that is involved in many neurological and neuromuscular diseases. Among others, a strong correlation was demonstrated between accumulated high level endonuclease III sensitive-sites and hyperglycemia in diabetes. Various laboratory-based DNA damage assays are currently in use. However, since their initiating concept is totally different, interest and efforts to miniaturize their fluidics and sensing methods reduced their efficiency tremendously. The **ultimate goal** of this multi-phase project is to commercialize the development of a portable DNA damage/repair quantitative detection system, easy to use by relatively unskilled personnel, yet providing a rapid-on-the spot screening result of tissue or blood cells for specific genomic DNA damage, and indicate susceptibility to a certain cancer or genetic disease. Such a device can then be made available to the doctor's office and even tomorrow's battlefield. This paradigm shift is possible by the application of silicon ministructures and microfluidics. Silicon microstructures and microfluidics technologies are moving out of the feasibility phase and into the application phase for the rapid analysis of complex DNA solutions. Our preliminary data using a concept of great ease and minimal fluidics demonstrate a chemical reagent probe specific for abasic sites, consistently and efficiently measured approximately 0.5 AP sites/10^5 bp in acid/heat stressed double stranded calf thymus DNA directly on the microtiter plate. Moreover, specific DNA oxidation products were determined when enzymatically processed with corresponding DNA repair endonuclease. **Hypothetically**, it is yet to be tested if similar efficiency could be obtained using recently developed silicon microstructures and application of microfluidics technologies for extracting clean DNA from complex biological samples towards automation. The **specific aim** of the current phase-I application is to quantitatively detect accurate abasic sites in genomic DNA of normal and pathological/free radical treated cells and tissues by combining the application of silicon microstructures and microfluidics technologies with the demonstrated chemical reaction and optical detection systems in a timely, efficient and cost effective manner.



ARP signal of calf thymus DNA containing specific Low AP site/10 000 bp determined by the Direct assay method using TMB substrate. The ARP signals are expressed as the change in absorbance at 650 nm after subtracting background readings obtained for control DNA.



ARP signal of Methoxyamine-treated calf thymus DNA incubated at physiological pH 7 and 37 C, undergoing spontaneous depurination at 0, 3, 6 and 9 days respectively; compared to Methoxyamine-treated calf thymus DNA incubated at pH 7 and 4 C incubated for 0, 3, 6, and 9 days.

	Slope x 10^{-3} (OD/min)	Slope x 10^{-3} (OD/min)	Slope x 10^{-3} (OD/min)
DNA (ug/mL)	**Direct Assay (ABTS)**	**Direct Assay (TMB). Methoxyamine** **Non-treated**	**Treated**
1.25	-	7.00	-
2.5	-	10.40	-
5.0	8.58	22.60	55.54
10.0	12.60	30.90	73.55
Lowest AP Quantified	0.3 AP site/10 000 bp	0.1 AP site/10 000 bp	0.05 AP site/10 000 bp

Table 1: Comparing the Sensitivity of Direct ARP Assay Methods

Brief Summary of the potential commercial applications of the research

The current developed concept is intended for patent filing and could be commercialized:

1. To develop simple and rapid assay kits for determining oxidative DNA damage in cells and tissues.
2. To develop simple and rapid enzyme-coupled assay kits for estimating DNA repair capacity in cells.
3. To develop a semi-automated DNA damage/repair assays using robotics for large thorough put samples in clinical application and populational research studies.
4. To develop a fully automated portable analysis system for rapid on-site detection of genotoxic, bio-warfare and toxicological agents for military personnel and clinical application.

Key Words: DNA damage, DNA repair, Abasic sites, Oxidative stress, Free radical damage, Silicon Microstructures and Microfluidics